

Metachromatic Agar-Diffusion Methods for Detecting Staphylococcal Nuclease Activity

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Based on the metachromatic property of Toluidine Blue O, three, convenient agar-diffusion methods have been developed that enable detection of the nuclease of *Staphylococcus aureus* at concentrations as low as 0.005 $\mu\text{g}/\text{ml}$ in agar and broth cultures. The interactions of agar and deoxyribonucleic acid with Toluidine Blue O are discussed.

A disc-overlay procedure was recently described by Lachica and Deibel (2) that permitted thermal stability determination of nuclease and facilitated demonstration of the close relationship among coagulase, enterotoxin, and thermostable nuclease production by *Staphylococcus aureus* (3). The method, however, has one shortcoming: the relatively complex manipulation necessitated by the use of an ultraviolet light and the sensitivity of the acridine orange fluorescence to quenching by proteins.

We sought to exploit the metachromatic properties of Toluidine Blue O (TB) to develop a simpler method for nuclease detection. The aptness of this approach was indicated by the development of a TB-flooding technique by Streitfeld and co-workers (6). Our efforts have yielded three convenient agar-diffusion procedures. The theoretical basis of these procedures is also discussed.

EXPERIMENTAL

Metachromasia. TB, a metachromatic dye, has been useful in histochemical studies because of its deviance from Beer's law when dissolved in water. It has an absorption maximum at 625 nm. At higher concentrations of the dye, the absorption peak is shifted to a shorter wavelength, 590 nm; in the presence of a polyanion, e.g., agar (5), the absorption maximum is at a still shorter wavelength, namely, 540 nm. Deoxyribonucleic acid (DNA), on the other hand, causes a slight shift to a higher wavelength (5). With TB from Matheson, Coleman and Bell Co. (certified biological

stain, C. I. no. 18) and a Bausch & Lomb Spectronic-20, the metachromatic effects of agar (Difco) and DNA (Difco) at pH 7 and 9 in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer were observed. Figure 1 illustrates the shifts of the absorption peak to a shorter (curve D, bright pink in color) and longer wavelength (curve B, dark purple) with the addition of agar and DNA, respectively. The longer wavelength peak was maintained when both DNA and agar were present (curve B). The substitution to a highly depolymerized DNA preparation from Nutritional Biochemicals Corp. resulted in a broad absorption peak (curve C). Thus, this preparation was eliminated from further study. Michaelis and Granick (5) suggested that, in the presence of agar, molecular aggregates of the dye are formed and that these are adsorbed to the agar. The behavior of TB in the presence of DNA may be analogous to the DNA-acridine complex reported by Lerman (4) in which the dye is intercalated between successive layers of the base pairs. Heretofore, the simultaneous interaction of DNA and agar with TB has not been demonstrated. Apparently, complexes of TB with agar are weaker than with nucleic acid and the latter shifts the absorption maximum to a higher wavelength.

TB-DNA-agar mixture. Preliminary experiments were conducted to determine the best combination of the TB-DNA-agar (TDA) mixture which would exhibit both a good color contrast and a large zone of nuclease activity (described below). The optimum conditions for *S. aureus* nuclease (1) were also taken into consideration. The following TDA formulation was chosen from among other combinations. To 1,000 ml of 0.05

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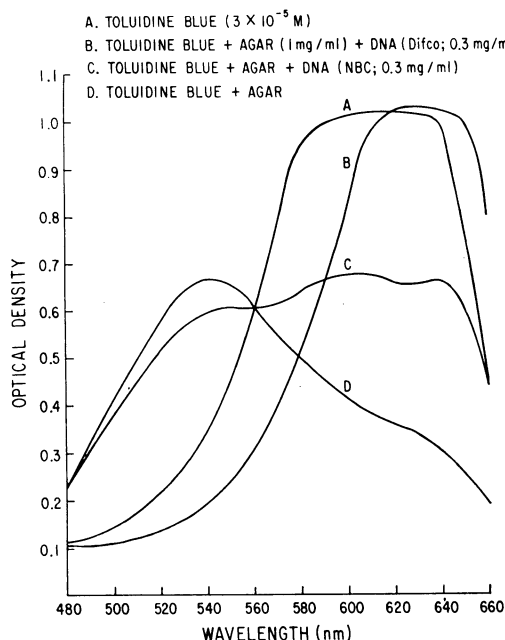


FIG. 1. Absorption curves of Toluidine Blue O in the presence of DNA or agar, or both.

M Tris buffer (pH 9.0) were added 0.3 g of DNA (Difco), 10.0 g of agar (granular, Difco), 1.0 ml of 0.01 M CaCl_2 , 10.0 g of NaCl, and 3.0 ml of 0.1 M TB. Before adding the TB solution, it is essential to boil the mixture until the DNA and agar are completely dissolved or melted. The mixture is divided into smaller volumes and stored at room temperature in rubber-stoppered flasks. For use, the TDA mixture is simply remelted.

An important feature of the TDA mixture is its remarkable stability; sterilization is not necessary even when stored at room temperature for as long as 4 months. Moreover, satisfactory results are obtained even when the mixture is subjected to several melting cycles. The stability of the TDA mixture may be attributed to the inhibitory property of TB towards gram-positive bacteria, especially the sporeformers, and the stability of the TB-DNA complex to heat.

Colony overlay procedure. Three agar-diffusion methods were designed to attain facility and versatility in determining nuclease activity. The colony overlay procedure was designed to detect nuclease activity in semisolid cultures and consists of overlaying a nutrient agar plate containing grown colonies with molten TDA mixture. After incubation for 1 to 4 hr at 37°C, hydrolysis of DNA is indicated by bright pink haloes around the colonies. Several strains of *Micrococcaceae*

(Appl. Microbiol., *in press*) were grown in Brain Heart Infusion (BHI) agar and subjected to the overlay technique. For most strains of *S. aureus*, the zone of nuclease activity is usually observed after 1 to 2 hr of incubation. Some strains of *S. epidermis* and micrococci also manifested nuclease activity, usually after an incubation period of 3 to 4 hr.

Microslide method for semisolid cultures. This method was designed to determine the thermal stability of the nuclease elaborated in agar cultures. A TDA microslide is made by pipetting 3.0 ml of molten TDA mixture to a microscope slide or a plastic immunoplate (Hyland). TDA slides may be prepared 8 days before use if precautions are made to minimize desiccation. With prolonged storage, the TDA begins to turn pink, causing difficulty in the color contrast. Remelting "recharges" the TDA mixture. To check for

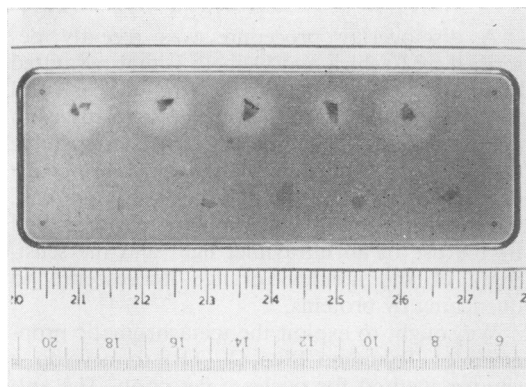


FIG. 2. Samples of five strains of *Staphylococcus aureus* from previously heated semisolid cultures (15 min of boiling) that were placed on the top portion of the Toluidine Blue-DNA-agar slide show reddish haloes of nuclease activity. Five strains of *S. epidermidis* were negative under similar conditions (bottom).

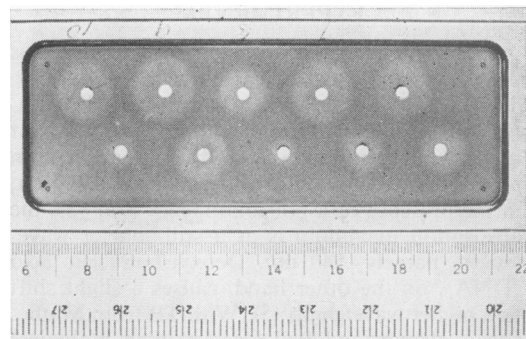


FIG. 3. Varying pink zone sizes of DNA hydrolysis were exhibited by samples of *Staphylococcus aureus* cultures after incubation for 3 hr at 37°C.

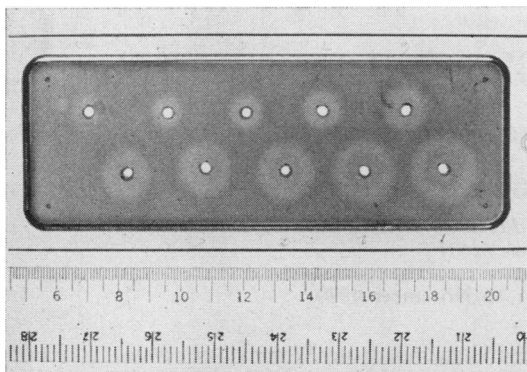


FIG. 4. Diameters of zones of hydrolysis of DNA varied directly with the concentrations of purified nuclease (*Staphylococcus aureus*; Worthington). The range of nuclease concentration was 2.0 to 0.005 $\mu\text{g/ml}$, the volume added to each well was constant at 2.0 μliters , and incubation of the Toluidine Blue-DNA-agar slide at 37 C was continued for 4 hr.

thermostable nuclease, an agar block supporting an isolated colony is cut out and transferred with an inoculating needle to a test tube (11 by 75 mm). After 15 min in a boiling-water bath, the gelled agar is fished out of the tube and placed onto the TDA slide. Figure 2 illustrates five cultures positive (top) and five cultures negative (bottom) for thermostable nuclease after 4 hr of incubation at 37 C.

Microslide method for broth cultures. The TDA slide is also used to determine nuclease activity in broth cultures. Ten or 12 wells, 2 mm in diameter, are cut in the slide and the agar plugs are aspirated with a metal cannula attached to vacuum. Employing a small platinum loop which is simply flamed before and after use, the wells are filled with heated (15 min of boiling) and un-

heated samples of broth cultures of strains of *Micrococcaceae*. Again, heat-stable nuclease activity is signalled by bright pink zones of DNA hydrolysis, discernible after 2 to 4 hr of incubation at 37 C. Nuclease activity varies with different strains of *Micrococcaceae* (Fig. 3). Although some strains of *S. epidermidis* and *Micrococcus* sp. exhibited nuclease activity before heating, none had nuclease activity after the heat treatment (4).

Preliminary experiments indicate that the microslide technique is amenable to quantitative assay. Zones of nuclease activity obtained with a series of dilutions (BHI broth used as diluent) of purified nuclease of *S. aureus* (Worthington) from 2.0 $\mu\text{g/ml}$ down to as little as 0.005 $\mu\text{g/ml}$ are depicted in Fig. 4.

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